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Expression and regulation of Spätzle-processing enzyme in *Drosophila*

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Abstract The *Drosophila melanogaster* Toll receptor controls embryonic dorsal-ventral axis formation and is crucial for the innate immune response. In both cases, Toll is activated by the enzymatically cleaved form of its ligand Spätzle (Spz). During axis formation, Spz is cleaved by the maternally provided serine protease Easter while the Spätzle-processing enzyme (SPE) activates Spz after infection. We confirm the role of SPE in immunity and show that it is a zygotic gene specifically expressed in immune tissues implying that the dual activation of Spz is achieved by differential spatiotemporal expression of two similar but distinct serine proteases.

Keywords: RNA interference; Serine protease; Fat body; Lymph gland; Innate immunity; Spätzle-processing enzyme; Toll; *Drosophila*

1. Introduction

Serine proteases form a large group of peptidases involved in vital processes such as digestion, blood coagulation, fertilization, immune response and embryonic development. When organized in proteolytic cascades they can mediate a rapid and localized response to physiological or foreign stimuli. Two of those cascades culminate in activation of the Toll receptor by the cytokine-like polypeptide Spz. During dorsal-ventral axis formation of the *Drosophila* embryo, local activation of Toll establishes a morphogenetic gradient of the Rel/NF-κB transcription factor Dorsal, which determines the region-specific expression of zygotic patterning genes (reviewed by [1]). The maternally contributed Easter is the terminal protease of the cascade that leads to activation of Spz. Easter is required only during the syncytial blastoderm stage as demonstrated in rescue experiments by mRNA injection and with temperature sensitive alleles [2].

In adult flies, Spz activates Toll in response to Gram-positive bacteria and fungal infections. The activation results in humoral reactions based on production of antimicrobial peptides (AMPs) [3]. The AMP *Drosomycin* (Drm) is mainly induced by this pathway, while other AMPs, such as *Diptericin* (Dpt), are strongly induced by Gram-negative bacteria and regulated by the immune deficiency (IMD) pathway (reviewed in [4]). Only recently, the serine protease that activates Spz in immunity, SPE (Sp4, CG16705) has been comprehensively characterized [5].

We have generated RNA interference (RNAi) transgenic flies targeting several proteases reported to be upregulated upon infection [6–8]. The RNAi flies were tested for susceptibility to infection. Ubiquitous drivers were used when the silencing generates viable flies (CG16705-SPE, CG3505, CG3066 and CG2045) and immune-specific drivers in the case of lethality with ubiquitous depletion (CG9733, CG6639). We observed that mutant lines targeting *SPE* had impaired resistance to the Gram-positive bacteria *Enterococcus faecalis* while the resistance to infection with the Gram-negative bacteria *Pseudomonas aeruginosa* was unaffected, indicating a role of *SPE* in the Toll signaling pathway.

We further show that *SPE* is constitutively expressed during embryogenesis in the developing fat body, the functional analog of the vertebrate liver, and in the lymph glands, the larval hematopoietic organ. In larval stages, expression is mainly confined to the fat body suggesting that SPE is secreted to the hemolymph. The expression pattern of *SPE* demonstrates that the two proteases that activate Spz, Easter and SPE, although enzymatically redundant, execute distinct physiological functions due to different spatiotemporal expression (this study) and due to distinct upstream activating proteases [5].

We address the regulation of *SPE* and show that the inducible expression of *SPE* is controlled by the Toll pathway establishing a positive feedback loop that reinforces the infection cues. We further show that depletion of *SPE* impairs the induction of Drm and this effect is exerted upstream of the receptor.

2. Materials and methods

2.1. Drosophila stocks

Fly stocks were obtained from the Bloomington Stock Center unless otherwise specified. Oregon^{R} and w^{1118} were used as wild-type controls. Mutant flies were: spätzle^{rm7}/ spätzle^{rm7} (spz); relish^{E20}/ relish^{E20} (rel); key^{1}/ key^{1} (key); tub^{1}/ tub^{2} (tube) and Tl^{10b}. The Gal4 driver line Act-Gal4/CyO was combined with the RNAi transgenic line 16705-RNAi generating ubiquitous depletion of the SPE transcript.

2.2. Transgenic RNAi

Genomic and cDNA sequences in opposite direction were cloned into pUAST to produce a dsRNA against CG16705 as described in [9]. The genomic fragment including the upstream flanking sequence and the two first introns was amplified using the primers (5′-GGGCGGCCGCCCAATGCATCGGGAGAG-3′) and

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Abbreviations: AMP, antimicrobial peptide; Dpt, Diptericin; Drm, Drosomycin; IMD, immune deficiency; RNAi, RNA interference; SPE, Spätzle-processing enzyme; Spz, Spätzle

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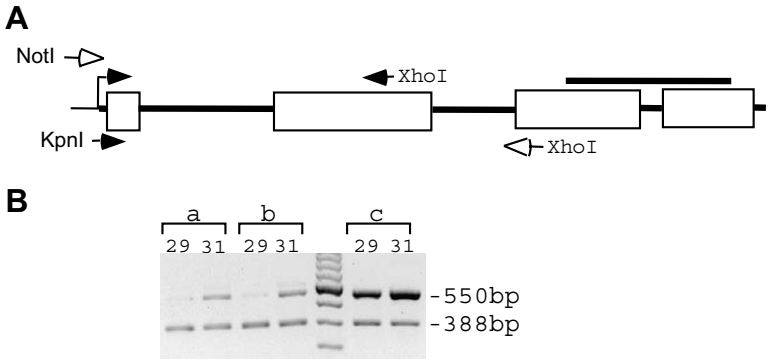

Fig. 1. RNAi strategy and depletion of *SPE* transcripts. (A) Exon-intron structure of *SPE*. Boxes represent exons. Primers and restriction sites used to construct RNAi transgenes are indicated by arrows. Hollow arrows denote primers used to amplify genomic DNA. Filled arrows denote primers used to amplify cDNAs. The line on top represents the probe used for in situ hybridization as well as the product of the RT-PCR for expression quantification. (B) Quantification of the *SPE*-depletion. RT-PCR products of *SPE* (550 bp) and of the internal control rp49 (388 bp) amplified for 29 cycles or 31 cycles. Genotypes: *Act*-Gal4/CyO;16705-RNAi/16705-RNAi (lanes a); *Act*-Gal4/CyO; 16705-RNAi/ TM3-*Sb* (lanes b); *Act*-Gal4/CyO; +/+ (lanes c).

(5′-GGCTCGAGGTTTCAGAAAAAACTGAAAGG-3′), and the cDNA fragment using the primers (5′-GGGGTACCCATAGCGACGAGATGGCT-3′) and (5′-GGCTCGAGTCCATGATATTGCCCCG-3′), (Fig. 1A). Subcloning and transformation was done as described in [10]. Eight independent transgenic lines were recovered.

2.3. Semi-quantitative RT-PCR

Quantitative estimation of the knockdown effect was performed by RT-PCR. One μg of total RNA extracted from adult flies was reverse transcribed using oligo-(dT) and 1/20 of the reaction was amplified for 29 or 32 cycles with the primers (5′-AACTCGCGCTACGTCCTGACC-3′) and (5′-ATAGGCACCATAAGGGGACCG-3′) targeting the 3′ end of the gene and the primers (5′-GACCATCCGCCCAGCATACAGGC-3′) and (5′-GAGAACGCAGGCGACCGTTGG-3′) targeting the ribosomal protein-encoding gene *rp-49* as control. In order to compensate the distinct abundance of transcripts, primers for *SPE* were used at 200 nM and 40 nM for rp-49.

Induction of AMPs was tested by multiplex PCR of random primed reverse transcription. In addition to the internal primers for rp-49 (40 nM), the reaction included primers for Drm (100 nM) (5′-CGTGAGAACCTTTTCCAATATGATG-3′) and (5′-GAATATGTGTAAGTAGTGGAGAG-3′) and Dpt (100 nM) (5′-ACTTTGCTGCGCAATCGCTTCTAC-3′) (5′-CCATATGGTCCTCCCAAGTGC-3′).

2.4. Immune challenge

Septic injury was performed by pricking the thorax of adult flies (aged 2–4 days) with a needle dipped into a concentrate (O.D. 200) of overnight bacterial culture. For survival experiments, 20 flies of each sex per vial were infected and incubated at 29 °C. Vials were positioned upside down to avoid the sticking of injured flies to the food and to facilitate the counting of dead flies.

2.5. In situ hybridization

Single stranded DNA probes were generated by asymmetric PCR. 200 ng of the 550 bp band corresponding to the 3′ end of the *SPE* transcript was labeled using a digoxigenin-labeling kit (Roche Applied Science). The reverse primer was used to generate the anti-sense probe. The forward primer was used to produce the sense probe for negative control experiments. In situ hybridizations were carried out according to Tautz and Pfeifle [11].

3. Results and discussion

3.1. Inherited RNAi efficiently knocks down SPE transcript levels

In order to quantify the reduction of *SPE* transcription in silenced lines, we performed RT-PCR on RNA from adult flies induced with the ubiquitous driver *Act*-GAL4 (Fig. 1B). One copy of the RNAi transgene reduced transcript levels by 94% while two copies reduced the expression by 97%. The driver line, as well as the parental line w^{1118}, showed constitutive expression of *SPE* in the absence of infection (Fig. 1B, lanes 29c and 31c and data not shown). Silencing of *SPE* did not cause lethality and a stock in which *SPE* was ubiquitously depleted could be maintained at RT. At 29 °C, however, this stock produced few viable progeny. This temperature sensitivity could explain the contradictory results on survival reported in two recent studies targeting *SPE* [5,12]. The rate of lethality after infection with the Gram-positive bacteria *E. faecalis* was increased in *SPE*-depleted flies, reaching 80% lethality 10 h post-infection, while the lethal rate was only 20% for wild-type flies.

3.2. SPE is expressed in immunological tissues

The spatial and temporal expression of *SPE* was examined by *in situ* hybridization to whole mount embryos and in dissected tissues. A maternal contribution of *SPE* was not detected. During embryogenesis, *SPE* is expressed in the lymph glands and in the fat body. Both organs are involved in the immune response. The lymph glands are the larval hematopoietic tissue and the fat body is the main source of circulating immune-related components. The fat body arises from segmentally repeated primary and secondary cell clusters located in the lateral and ventral mesoderm of parasegments (PSs) 3–12, respectively [13]. An additional dorsal cell cluster is located in the dorsal mesoderm of PS 13. The GATA transcription factor Serpent (spr) is the earliest fat body-cell marker but *spr* is also expressed in several other tissues [14]. Expression of *SPE* is first detected at early stage 11 at the posterior tip of the germ-band (Fig. 2A). During germband retraction, expression appears dorsally in PS 13 (arrowheads) and ventrally in PS 3–12 (stars) (Fig. 2C and E). The dorsal view at stage 12 (Fig. 2D) reveals expression at the tip of the retracting germband and in PS 13 (arrowhead). Tissue movements during embryogenesis suggest that cells at the tip of the germ-band may contribute to the posterior-most portion of the lateral fat body. Our results suggest that cells cluster in PS 13 make up dorsal fat body projections whereas the ventral

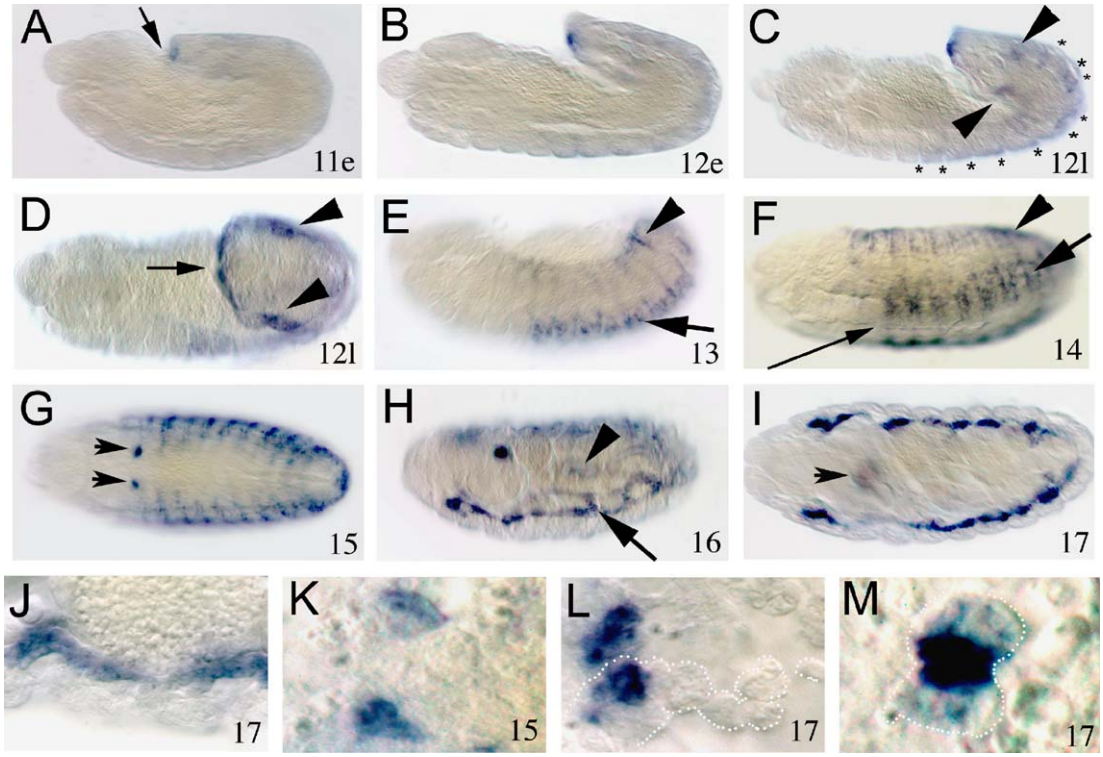

Fig. 2. Expression of *SPE* during embryogenesis. Embryos are shown in lateral view oriented anterior to the left and dorsal side up, except in F which is a ventral view and D,G,I,K,J and M, which are dorsal views. Developmental stages are according to [20] and indicated in the lower right corner. (A) Expression of *SPE* is first observed at early stage 11 at the posterior tip of the germband (arrow). During germband retraction (C), transcripts are detected ventrally in PSs 3–12 (stars) and dorsally and ventrally in PS 13 (arrowheads). (D) Dorsal view at late stage 12 showing two bilateral expression domains corresponding to the ventral PS 13 (arrowhead) and the posterior domain facing the amnioserosa (arrow). At stage 13, expression is detected in two ventrally (arrow) and dorsally located bilateral domains, with more intense expression in PS 13 (arrowhead). (F) At stage 14, the presumptive lateral fat body (arrow) is disconnected from the dorsal projection (arrowhead). No expression is detected at the ventral side (long arrow). (G) At stage 15 two intense bilateral spots are detected at the position of the developing lymph glands (arrowheads). (H) At stage 16, the lateral fat body (arrow) and the dorsal projection (arrowhead) are interconnected; the intensity of expression at the lymph glands is increased. (I) Expression in the lateral fat body at stage 17. The lymph glands are shown out of focus (arrowhead). (J) High magnification showing the fat body flanked by the epidermis and the gut. (K). Developing lymph glands at stages 15. (L and M) At stage 17, *SPE* is expressed in the first lobe of the lymph glands. Expression is restricted to the area adjacent to the dorsal vessel.

cell clusters give rise to the lateral fat body as postulated by Miller et al. [15]. At stage 14 (Fig. 2F), *SPE* expression is detected in two bilateral domains separated by an unstained zone running laterally. It is likely that the dorso-lateral domain (arrowhead) contributes to form the dorsal projections and the ventro-lateral domain (arrow) to the lateral fat body. At later stages the fat body forms an interconnected structure (Fig. 2H). The expression of *SPE* suggests that the dorsal projections and the lateral fat body arise independently and form an interconnected organ only late in embryogenesis.

The progenitors of the lymph glands have been mapped to the lateral mesoderm in segment A1 [16]. At stage 15, we detect transcription of *SPE* in this segment in bilateral clusters that very likely form part of the lymph gland primordia (Fig. 2G). After stage 15, the clusters move from a lateral position to their final location at both sides of the dorsal vessel (Fig. 2G,H and K,L). At the final stages of embryogenesis, expression in the lymph glands is restricted to specific areas flanking the dorsal vessel (Fig. 2L and M). This area has been described as containing "secretory cells" although no function has been assigned to them yet [17]. It is possible that SPE is secreted from these cells into the embryonic hemolymph. Alternatively these cells could differentiate into *SPE*-expressing mature hemocytes (see below). We could not detect any expression of *SPE* in the anlagen of the hematopoietic system in the head mesoderm or in circulating embryonic hemocytes where other fat body and lymph gland makers are expressed. Thus, *SPE* could be used as a specific marker for lymph glands and fat body early in development. Overall, transcriptional levels of *SPE* increase throughout embryogenesis.

At larval stages, *SPE* is highly expressed in the fat body and in mature hemocytes (Fig. 3A and D) with weak expression in the lymph glands (Fig. 3C). This tissue specific expression contrasts with the systemic immune response mediated by SPE. The target of SPE, Spz is localized in circulating hemolymph as demonstrated by rescue of the immune response in *spz* mutants by transfer of wild type hemolymph [18]. Thus, it is likely that both inactive proteins are circulating in the hemolymph. After infection, the cleavage cascade spreads through the hemolymph and activates Toll in the fat body leading to systemic production of effector molecules such as AMPs but also to the upregulation of *SPE* transcription (Fig. 5 and below).

3.3. Expression of SPE is increased after immune challenge and is regulated by the Toll pathway

SPE is rapidly upregulated in response to infection and we could detect a twofold increase of transcription only 3.5 h after septic injury (Fig. 4B). This upregulation is absent in Toll

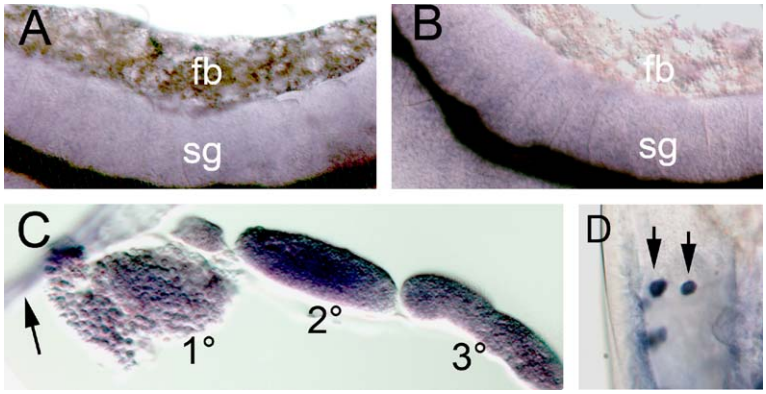

Fig. 3. Expression of *SPE* in larval tissues. (A) *SPE* transcripts in the fat body (fb), that is attached to the salivary glands (sg). (B) In situ hybridization with the *SPE* sense probe does not generate a signal in the fat body. The lumen of the salivary glands is non-specifically stained. (C) Expression in the lymph gland lobes (1°–3°). The dorsal vessel is indicated by an arrow. (D) Blood cells showing expression of *SPE* attached to an imaginal disc.

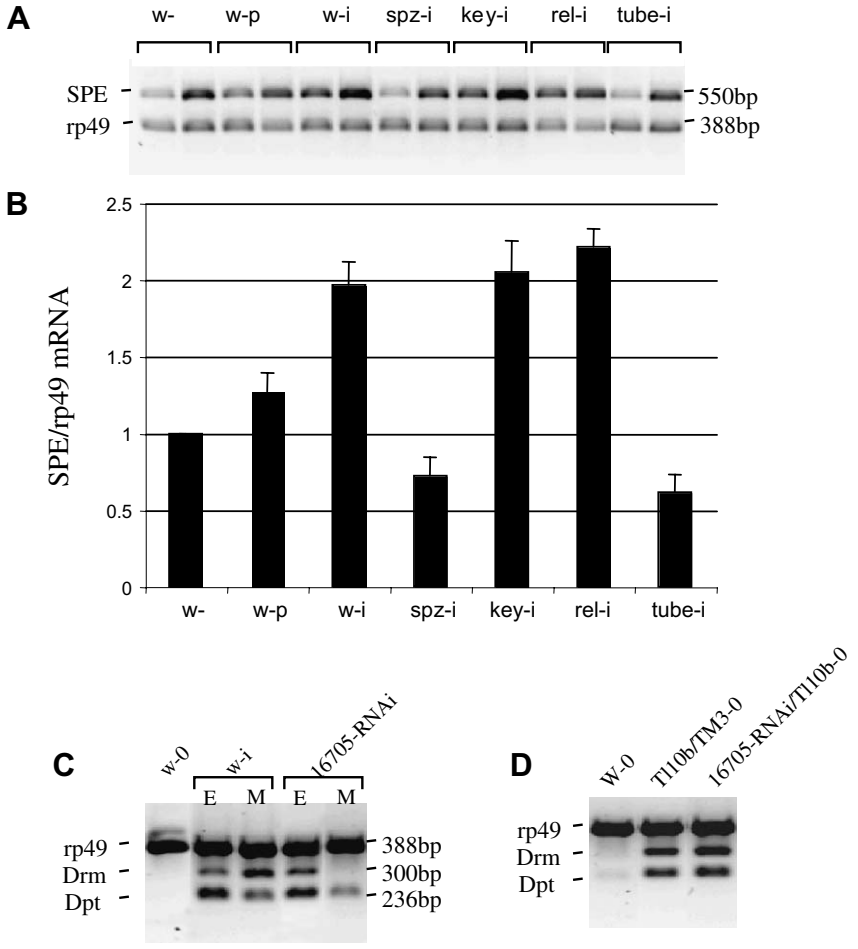

Fig. 4. SPE acts upstream of Toll and is transcriptionally regulated by the Toll pathway. (A) Estimation of *SPE* mRNA in flies of different genotypes by RT-PCR. Total RNA was extracted 3.5 h after clean pricking (w-p), infection with a mixture of *E. coli* and *M. luteus* (w-i, spz, key, rel, tube) or untreated flies (w-). The amount of *SPE* (550 bp) and the rp49 internal control (388 bp) were quantified after 28 (left well) or 31 cycles (right well) of RT-PCR. (B) Relative amounts of *SPE* mRNA from three independent experiments and standardized against rp49. (C) Gel electrophoresis showing multiplex RT-PCR, after amplification of Drm, Dpt and the internal control, rp49. Adult flies of each genotype were non-infected (0) or challenged with *E. coli* (E) or *M. luteus* (M). RNA was prepared 24 h after challenge and reverse transcribed using random hexamers. (D) The constitutive induction of AMPs in Tl^{10b} mutants is not blocked by 16705-RNAi, indicating that SPE acts upstream of the receptor.

pathway mutants (spz and tube) but not significantly affected in mutants of the IMD (key and rel) or JNK pathway (hep) (Figs. 4B and 5 and results not shown) suggesting that SPE is induced by Toll signaling upon infection. The constitutive

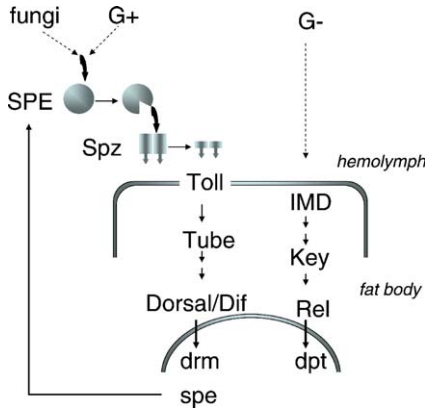

Fig. 5. Model for the regulation of SPE during immune response. Induction of SPE transcription depends on Toll signaling. SPE secretion to the hemolymph in response to infection activates Spz, thereby generating a positive feedback loop. Mutants and effector molecules used in this study are indicated in their respective pathways.

transcription of *SPE* in untreated flies (w-), and injured non-infected flies (w-p) in Fig. 4B, is higher than in challenged Toll pathway mutants, suggesting that the constitutive transcription could also be dependent on this pathway.

3.4. SPE acts upstream of Toll and is required for the production of antimicrobial peptides after infection by Gram-negative bacteria

We further addressed whether the expression of antimicrobial peptides was affected in *SPE*-depleted flies. Induction of Drm and Dpt was analyzed after infection with either Gram-negative (*E. coli*) or Gram-positive bacteria (*Micrococcus luteus*) (Fig. 4C). Wild type flies induced mainly Dpt when infected with *E. coli* and Drm when infected with *M. luteus*, while neither AMP was detectable in non-challenged flies. In *SPE*-depleted flies, the response to *E. coli* infection is similar to wild type flies but the production of Drm is blocked after infection with *M. luteus*, indicating that Toll pathway activity was reduced.

In order to determine the epistatic relation between *SPE* and Toll, flies carrying a constitutively active allele of *Toll* (Tl^{10b}) were depleted for *SPE* (Fig. 4D). As expected, Tl^{10b} mutants constitutively express Drm but we and others [19] observed that the mainly IMD-regulated AMP, Dpt, is also upregulated in untreated flies. The production of AMPs was not suppressed when *SPE* was depleted in uninfected flies, indicating that SPE acts upstream or in parallel to the receptor. This is consistent with the recent finding that Spz is in fact the target of SPE during Toll dependent immune activation [5].

In conclusion, we confirm the role of SPE in immunity. We further show that *SPE* is a zygotic gene constitutively expressed in immunological tissues acting upstream of Toll and feedback regulated by the same pathway.

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